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Higher export rate of homocysteine in a human endothelial cell line than in other human cell lines

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Abstract

Even mild hyperhomocysteinemia is associated with premature vascular disease. Despite the growing evidence that plasma homocysteine is a cardiovascular risk factor, the mechanism behind the vascular injuries is still unknown. Information about the metabolism of homocysteine is, therefore, essential for an understanding of its role in atherogenesis. In the present study we have, therefore, investigated the export mechanism of homocysteine. In HeLa cell lines the release of homocysteine was found to be a continuous process, which was increased in the presence of copper ions. High cell density led to a lowered release of homocysteine, probably due to a more extensive metabolism of the intracellular homocysteine. It was also found that HeLa cells were able to take up extracellularly released homocysteine and use it in the cellular metabolism. The ratio between intracellular homocysteine and the total amount of homocysteine is a measure of the ability of the cell to export the intracellularly produced homocysteine. The ratio also reflects the reuse of extracellular homocysteine. Under basal conditions, endothelial cells exported most of the intracellularly produced homocysteine and exhibited a very low concentration of homocysteine intracellularly, low reusage of exported homocysteine and consequently a low ratio in comparison with HeLa and hepatoma cell lines. After addition of homocysteine, all cell lines exhibited similar ratios. Thus, the intracellular homocysteine concentration in endothelial cells is more influenced by the extracellular concentration of homocysteine than is the intracellular concentration in HeLa and hepatoma cells. It may be speculated that this phenomenon could be associated with an increased sensitivity of endothelial cells to homocysteine and explain the association between hyperhomocysteinemia and vascular disease.

Keywords: Copper ion; Cysteine; Glutathione; Homocysteine; Human cell line

1. Introduction

Homocysteine, a sulfhydryl amino acid, is the demethylated derivative of methionine [1]. Greatly elevated plasma levels of homocysteine are found in subjects with homocystinuria [1]. These patients exhibit early arteriosclerosis, arterial and venous thrombosis. Approximately 30 epidemiological studies published during the last ten years show that even mild hyperhomocysteinemia is associated with premature vascular disease [2–8]. Despite the growing evidence that plasma homocysteine is a cardiovascular risk factor, the mechanism behind the vascular injuries is still unknown. Information about the metabolism of homocysteine is therefore essential for an understanding of its role in atherogenesis, thereby enabling a modulation of that risk.

Adenosyl-methionine is the immediate precursor of homocysteine and is the principal methyl donor

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in mammals [1]. After a methyl transfer reaction (transmethylation), adenosyl-homocysteine is hydrolysed by *S*-adenosyl-homocysteine hydrolase to homocysteine and adenosine. Homocysteine may either be catabolized in the transsulfuration pathway via cystathionine to cysteine or remethylated back to methionine. Cysteine is a precursor to glutathione [9]. All these three thiols are thus metabolically related. Glutathione, a thiol containing tripeptide, provides cells with their reducing power and maintains the sulfhydryl groups of proteins and other compounds in reduced form. It protects the cells against oxidative damage and other types of injury such as metal toxicity [9].

The intracellular concentration of homocysteine seems to be strictly regulated by export mechanisms [10]. Under normal conditions cells export considerable amounts of the intracellularly produced homocysteine. Perturbation of its intracellular metabolism may lead to accumulation of homocysteine intracellularly resulting in a further increase of its export. Thus, the export occurs at a rate reflecting the balance between intracellular production and further metabolism. This mechanism is the biological basis for accumulation of homocysteine in extracellular fluids like plasma and urine in patients with inborn errors of homocysteine metabolism [9], and with folate or cobalamin deficiency [11]. In experimental studies, enhancement of cellular homocysteine export is seen when methotrexate or nitrous oxide, acting as inhibitors of remethylation, are added [12,13]. Likewise, an increased concentration of methionine in cell culture medium induces a marked enhancement of homocysteine export from most cells [14], whereas addition of folates [15] lowers the export of homocysteine. The enzyme responsible for the interconversion of adenosyl-homocysteine to homocysteine, *S*-adenosyl-homocysteine hydrolase, has been the target for the cytostatic action of purine analogues [16]. Inhibition of the formation of homocysteine by such compounds is followed by a decrease in homocysteine export [17].

We have developed a procedure for determination of thiols [18]. We have recently shown that even low concentrations of copper ions (0.1–1 μmol/l) had a profound effect on homocysteine export in a HeLa cell culture system [19,20]. Copper ions increased the release of homocysteine into the medium, possibly by interaction with *S*-adenosyl-homocysteine hydrolase [19,20]. Furthermore, the addition of copper ions seemed to increase the oxidative stress in the cells as indicated by a decrease of reduced thiol species both intra- and extracellularly [19]. The addition of copper ions might therefore disturb the export mechanism of homocysteine in several ways (e.g. by interfering with *S*-adenosyl-homocysteine hydrolase or by decreasing the proportion of reduced thiol fractions). In the present study we have focused on the export mechanism of homocysteine in relation to time, cell density and copper ions. Cysteine and glutathione were also determined in order to elucidate their possible interaction with homocysteine export. In order to investigate potential cell type differences in the export mechanism of homocysteine, several human cell lines were examined.

2. Materials and methods

2.1. Cell culture

The established HeLa cell line, human hepatoma cell line (HepG2) and a human transformed endothelial cell line (ECV 304) were obtained from American type culture collection, Rockville, MD. The cells were cultured at 37°C in 75 cm^2 flasks, in 12 ml of RPMI 1640 (Gibco Laboratories, Santa Clara, CA) containing 10% fetal calf serum (Gibco laboratories). The cells were grown in humidified air with 5% CO_2. The cells were routinely screened for and shown to be free from mycoplasma using a kit with a specific DNA-probe from Gen-Probe, San Diego, CA. New medium with or without test substances was added at the start of the experiments. In some experiments 24 h of incubation was used and in other experiment the cells were maintained in the same medium for 3 days or the medium was changed daily for 3 days. The substances (DL-homocysteine, $CuSO_4$) added (in a volume of 100 μl) to the cell culture experiment were prepared in 1 mmol/l of HCl immediately before addition. Both chemicals were bought from Sigma, St. Louis, MI.

2.2. Methods

After the experiments the cells were detached with

a rubber policeman. The cells and medium were immediately centrifuged (2000×*g* for 5 min). Assay of the total thiols was as follows: 70 μl of 0.3 M Tris buffer pH 8.5 and 30 μl of 0.1 M dithiothreitol (DTT) were added to 400 μl of medium. The cells were immediately washed and centrifuged (2000×*g* for 5 min) twice with 2 ml ice-cold phosphate buffered saline pH 7.3 (containing 8.6 g KH_2PO_4, 48 g $Na_2HPO_4 \cdot 2H_2O$ and 25 g NaCl per liter). Thereafter, the cells were homogenized in 500 μl of 10 mM phosphate buffer pH 8.0 and 30 μl 0.1 M DTT. The homogenate and medium were incubated at 37°C for 15 min and then 100 μl of 15% sulfosalicylic acid were added. After 30 min at 4°C, centrifugation (12 000×*g* for 5 min at 4°C) was performed and the supernatant was frozen at −70°C until analysis, not more than 2 days later. This procedure measures the total amount of thiol moieties, whether disulfide-bound or not. The determinations of the thiols were carried out as previously described [18]. In short, the analyses were performed with a high-performance liquid chromatographic method, which utilized isocratic reversed-phase ion-pair liquid chromatography at pH 2.4 and postcolumn derivatization with 4,4′-dithiopyridine and colorimetric detection at 327 nm. Total imprecision (coefficient of variation, CV) for these determinations was below 5% for all thiols measured. To test the influence of matrix differences in cell homogenate and the calibration sample, additions of cysteine, homocysteine and glutathione to cell homogenates and media were performed. The recoveries of added thiols were between 94 and 102%. The cellular contents of thiols are expressed as the total amount (nmol) or amount per mg cell protein. In the medium thiols are expressed as the amount present in the total volume of medium (12 ml) or nmol/ml medium. Protein was analyzed according to Lowry et al. [21] and cell growth was measured as the increase of cell protein.

2.3. Statistics

Results are expressed as mean and S.D. Statistical significance between the different groups was assessed by the Wilcoxon rank test; $P < 0.05$ was considered significant. Spearman's rank correlation coefficients were calculated to test for monovariate relationship between different variables.

3. Results

3.1. Homocysteine release in HeLa cell cultures in relation to time, copper ions, and fetal calf serum

After an initial equilibrium between intra- and extracellular homocysteine fractions (within 3 min), there was a continuously increasing concentration of extracellular homocysteine with time both in the presence and absence of copper ions during 24 h of incubation (Table 1). Cell cultures with and without added copper ions exhibited a decreased release of homocysteine into medium when the concentration of fetal calf serum was decreased (Table 2). Table 3 shows that even addition of 1 μmol/l of copper ions increased the release of homocysteine to medium compared with cell cultures without any copper addition both after daily change of medium for 3 days and after 3 days in the same medium. The release of homocysteine was almost maximal already after the addition of 10 μmol/l of copper ions.

Table 1
The variation of intra- (nmol/mg protein) and extracellular (nmol/ml) concentration of homocysteine with time in HeLa cell cultures incubated for 24 h with and without the addition of 10 μmol/l of copper ions[a]

	3 min	1 h	3 h	9 h	24 h
Cultures without any addition ($n=4$)					
Cell Hcy	1.2 ± 0.3	1.1 ± 0.3	1.3 ± 0.4	1.3 ± 0.3	1.5 ± 0.4
Medium Hcy	0.5 ± 0.2	1.0 ± 0.4	1.7 ± 0.5	2.8 ± 0.6	5.5 ± 0.9
Cultures with addition of 10 μmol/l of copper ions ($n=4$)					
Cell Hcy	1.3 ± 0.4	1.1 ± 0.3	0.9 ± 0.4	1.3 ± 0.4	1.3 ± 0.5
Medium Hcy	0.5 ± 0.2	1.1 ± 0.2	1.8 ± 0.4	5.9 ± 0.9*	12.1 ± 2.5*

*$p < 0.05$ compared with cell cultures without any addition.
[a]Medium without any contact with cells contained 0.1 nmol/ml of homocysteine.

Table 2
The variation of intra- (nmol/mg protein) and extracellular (nmol/ml) concentration of homocysteine and total cell protein (mg) with the concentration of fetal calf serum (FCS) in HeLa cell cultures incubated for 24 h with and without the addition of 10 μmol/l of copper ions

	10% FCS	2% FCS	0% FCS
Cultures without any addition ($n=4$)			
Cell Hcy	0.9 ± 0.3	1.0 ± 0.3	0.9 ± 0.3
Medium Hcy	4.7 ± 0.9	2.6 ± 0.8#	1.8 ± 0.6#
Cell protein	4.3 ± 0.4	4.3 ± 0.6	2.9 ± 0.5#
Cultures with addition of 10 μmol/l of copper ions ($n=4$)			
Cell Hcy	0.9 ± 0.2	0.9 ± 0.3	0.8 ± 0.2
Medium Hcy	8.2 ± 1.9*	6.0 ± 1.2*	4.5 ± 0.9*#
Cell protein	4.6 ± 0.5	4.4 ± 0.3	2.9 ± 0.3#

*$P<0.05$ compared with cell cultures without addition of copper ions.
#$P<0.05$ compared with cell cultures incubated in 10% FCS.

3.2. Homocysteine release and cell density in HeLa cell cultures

The relation between thiol concentration and cell density, measured as total cell protein in HeLa cell cultures incubated for 24 h, was studied in 24 different experiments with cell protein varying between 0.8 and 7.9 mg per dish. The thiol concentrations used in the calculations are the mean from three dishes used in each experiment. Fig. 1 shows that the concentration of extracellular homocysteine (nmol/ml) decreases ($\rho=-0.82$; $P<0.001$) with increasing cell protein. From Fig. 1 it is also evident that the extracellular concentration of homocysteine is very low after 3 days in the same medium. The concentration

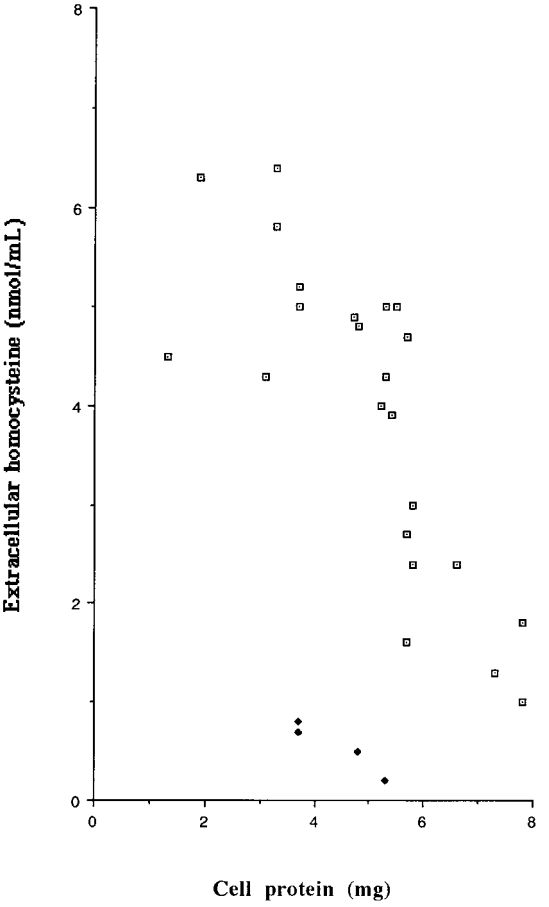

Fig. 1. The relation between extracellular homocysteine and total cell protein in HeLa cell cultures. The values shown are the mean from three dishes. Twenty-four experiments with different cell protein were performed. □ Daily change of the medium for 3 days. Analyses were performed on the third day. ◆ The same medium for 3 days. Analyses were performed on the third day.

Table 3
The variation of intra- (nmol/mg protein) and extracellular (nmol/ml) concentration of homocysteine and total cell protein (mg) with the concentration of copper ions (μmol/l) in HeLa cell cultures incubated for 3 days with daily change of medium or in the same medium for 3 days[a]

	Daily change for 3 days			Same medium for 3 days		
	C-Hcy	M-Hcy	C-protein	C-Hcy	M-Hcy	C-Prot
Controls	1.6 ± 0.4	4.4 ± 0.9	5.5 ± 0.3	0.9 ± 0.3	0.5 ± 0.2	4.6 ± 0.4
Cu, 1	1.6 ± 0.3	7.8 ± 1.6*	5.5 ± 0.4	1.0 ± 0.2	2.3 ± 0.6*	4.9 ± 0.4
Cu, 10	1.4 ± 0.5	8.3 ± 2.1*	5.4 ± 0.4	0.8 ± 0.3	7.2 ± 1.5*	4.8 ± 0.3
Cu, 50	1.5 ± 0.4	10.8 ± 3.0*	5.4 ± 0.5	0.7 ± 0.5	7.4 ± 1.2*	4.6 ± 0.3
Cu, 100	1.4 ± 0.4	10.9 ± 2.5*	5.6 ± 0.5	0.9 ± 0.4	7.5 ± 1.7*	4.7 ± 0.4

*$P<0.05$ compared with cell cultures without any addition (controls).
[a]The analyses were performed on the dishes from the third day. Four dishes are used for each experiment. Mean and S.D. are presented. C, cell; M, medium.

Table 4
The concentration of cell protein and total thiols in HeLa cell lines in the same medium for 1–3 days with and without (controls) the addition of 10 μmol/l of copper ions[a]

	C-Protein (mg)	C-Hcy (nmol/mg)	M-Hcy (nmol/ml)	C-Cys (nmol/mg)	M-Cys (nmol/ml)	C-GSH (nmol/mg)	M-GSH (nmol/ml)	M-CGz (nmol/ml)
Controls								
24	2.8 ± 0.4	0.9 ± 0.3	6.8 ± 0.9	14 ± 5	280 ± 21	171 ± 22	8.4 ± 1.8	5.5 ± 1.0
48	3.9 ± 0.4	1.3 ± 0.5	5.0 ± 0.8	22 ± 6	228 ± 23	87 ± 19	5.9 ± 1.2	8.9 ± 2.1
72	3.7 ± 0.3	0.8 ± 0.3	0.8 ± 0.4	25 ± 5	202 ± 32	88 ± 23	5.2 ± 1.8	8.0 ± 1.2
Copper ions								
24	3.2 ± 0.5	0.8 ± 0.2	8.5 ± 1.9*	11 ± 4	290 ± 20	154 ± 34	9.8 ± 2.1	9.0 ± 1.1
48	4.4 ± 0.3	1.0 ± 0.3	10.4 ± 2.1*	14 ± 4*	260 ± 18*	85 ± 17	8.7 ± 1.5*	16.1 ± 3.1*
72	3.8 ± 0.6	0.9 ± 0.2	8.0 ± 1.6*	15 ± 6*	250 ± 26*	78 ± 18	7.6 ± 1.6	21.3 ± 4.6*

*$P < 0.05$ compared to controls.
[a]Means and S.D. are presented. C, cell; M, medium; Hcy, homocysteine; Cys, cysteine; GSH, glutathione; CG, cysteinylglycine. Cell protein is expressed as mg protein per dish. Eight dishes are used for each experiment. Cell protein at the start of the experiment varied between 1.7 and 2.1 mg per dish.

of intracellular homocysteine (nmol/mg) showed no significant relation to cell protein.

In these correlation studies cysteine and glutathione were also studied in an attempt to further elucidate the metabolism of homocysteine. The concentration of intracellular cysteine increases ($\rho = 0.71$; $P < 0.001$) and the concentration of extracellular cysteine decreases ($\rho = -0.67$; $P < 0.01$) with increasing cell protein. The concentration of intracellular glutathione decreases with increasing cell protein ($\rho = -0.68$; $P < 0.01$), whereas extracellular glutathione did not show any significant relation to cell protein.

3.3. Homocysteine release in HeLa cell cultured for 1–3 days

Cells with and without addition of copper ions were incubated in the same medium for 1–3 days and homocysteine, cysteine and glutathione were determined for each day (Table 4). There were no changes in intracellular homocysteine between different days in cells cultured with or without the addition of copper ions. Extracellular homocysteine was higher in the presence of copper ions especially on day 3, where the control cell cultures only exhibited a very low concentrations of extracellular homocysteine. These findings for extracellular homocysteine concentrations on day 3 differ from those of the other two thiols. Also the two other thiols investigated in these experiments showed different concentrations without and with addition of copper ions. The concentration of intracellular cysteine was lowered and the concentration of extracellular cysteine was increased in the presence of copper ions. The release of glutathione was higher in the presence of copper ions especially if cysteinylglycine, the degradation product of glutathione, was included in the estimation of total glutathione release. Intracellular glutathione decreased with time but exhibited similar concentrations both with and without the addition of copper ions.

3.4. The ratio between intracellular and total homocysteine in different human cell lines

The ratio between intracellular and total amount of homocysteine (the sum of the intracellular and

Table 5
The relation of cell protein and the ratio between the amount of intracellular homocysteine and the total amount of homocysteine in HeLa cell cultures incubated for 24 h[a]

Total cell protein (mg)	Ratio (%)
1.3 ± 0.3	1.4 ± 0.2
3.7 ± 0.5	3.0 ± 0.5
5.2 ± 0.4	5.2 ± 0.9
5.4 ± 0.4	6.4 ± 1.4
5.7 ± 0.5	9.1 ± 1.7
7.3 ± 0.4	13.1 ± 2.3

[a]Mean and S.D. are given. Four dishes are used in each experiment.

extracellular amount of homocysteine) in the cell culture after 24 h of incubation varied between 1.4–13.1% depending on cell density in HeLa cell cultures (Table 5). The ratio, using the same cell density (cell protein 4–5 mg), was about 6% for HeLa and hepatoma cell cultures whereas the ratio for endothelial cells was about 1% (Table 6). After the addition of 100 μmol/l of homocysteine 1.0–1.3% of the total amount of homocysteine was present intracellularly after 24 h of incubation in all three cell lines.

Due to these findings, the release of homocysteine in endothelial cell cultures was investigated in the same way as reported for HeLa cell cultures. The endothelial cells behaved as HeLa cells in many aspects. They showed a continuously increasing concentration of extracellular homocysteine with time and a decreased concentration of extracellular homocysteine when the concentration of fetal calf serum was lowered. The addition of 1, 10 or 100 μmol/l of copper ions significantly increased the extracellular concentration of homocysteine after 24 h of incubation, but only with 15–25% compared to endothelial cell cultures without any addition. Endothelial cell cultures incubated in the same medium for 1 or 3 days showed a higher concentration of extracellular homocysteine in the presence of copper ions particularly on day 3, where the cell cultures without any addition exhibited a decreased concentration of extracellular homocysteine compared to day 1 (Table 7).

The most important difference between endothelial and HeLa cell cultures was the relation between cell density (measured as cell protein) and extracellular concentration of homocysteine. In HeLa cell cultures, extracellular concentration of homocysteine was decreased with increasing cell density (Fig. 1),

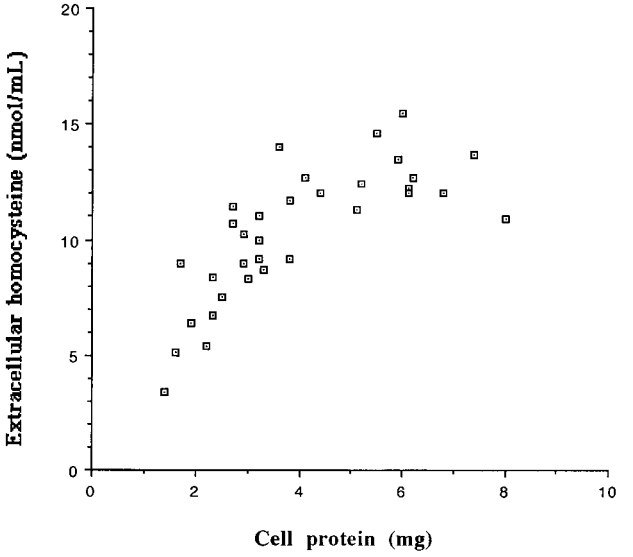

Fig. 2. The relation between extracellular homocysteine and total cell protein in endothelial cell cultures. The values shown are the mean from three dishes. Thirty-four experiments with different cell protein were performed. □ Daily change of the medium for 3 days. Analyses were performed on the third day.

Table 6
Intra- (nmol/mg protein) and extracellular (nmol/ml) concentration of homocysteine and the ratio (expressed as percentage) between the intracellular amount of homocysteine and the total amount of homocysteine in three different cell lines incubated for 24 h and the effect of addition of 100 μmol/l of homocysteine[a]

	HeLa cells	Hepatoma cells	Endothelial cells
Without addition			
C-Hcy	0.9 ± 0.3	1.3 ± 0.3	0.3 ± 0.1*
M-Hcy	5.2 ± 1.8	7.2 ± 2.2	12.0 ± 2.8*
Ratio	6.0 ± 2.1	6.3 ± 1.8	0.9 ± 0.3*
Addition of 100 μmol/l of homocysteine			
C-Hcy	2.6 ± 0.4	1.9 ± 0.2	2.4 ± 0.4
M-Hcy	88 ± 8	85 ± 7	87 ± 9
Ratio	1.3 ± 0.4	1.0 ± 0.2	1.0 ± 0.2

*$P < 0.05$ compared to HeLa cell cultures.
[a]Four dishes are used in each experiment. Cell protein was 4.2–5.3 mg per dish. Mean and S.D. are presented. C, cell; M, medium.

Table 7
The variation of intra- (nmol/mg protein) and extracellular (nmol/ml) concentration of homocysteine and total cell protein (mg) in endothelial cell cultures incubated for 1 or 3 days in the same medium with or without (controls) the addition of 10 μmol/l of copper ions[a]

	C-protein	C-Hcy	M-Hcy
Controls			
24	5.9 ± 0.7	0.21 ± 0.05	14.9 ± 2.2
72	5.2 ± 0.5#	0.31 ± 0.09	7.2 ± 2.8#
Copper ions			
24	5.8 ± 0.5	0.20 ± 0.05	17.0 ± 3.0*
72	5.0 ± 0.5#	0.32 ± 0.08	26.0 ± 5.5*

*$P < 0.05$ compared with cell cultures without any addition (controls).
#$P < 0.05$ compared with cell cultures after 24 h of incubation.
[a]Four dishes are used for each experiment. Mean and S.D. are presented. C, cell; M, medium.

whereas the opposite was observed for the endothelial cells ($\rho = 0.81$; $P < 0.001$; Fig. 2). The concentration of intracellular homocysteine in endothelial cell cultures showed no relation to cell protein. The ratios between intracellular and the total amount of homocysteine in the endothelial cell cultures, presented in Fig. 2, varied from 0.2% in the cell cultures with lowest amount of cell protein per dish to 1.2% in cell cultures with the highest amount of cell protein per dish.

4. Discussion

4.1. Findings in HeLa cell cultures

After an initial equilibrium between intra- and extracellular homocysteine fractions there was a continuous increase of extracellular homocysteine with time both in the presence and absence of copper ions. It was also evident that the presence of copper ions increased the release of homocysteine into the medium in cell cultures both with and without fetal calf serum. The increase of homocysteine export after addition of copper ions is in agreement with earlier findings [19,20].

The thiols investigated showed a complex pattern when cell density (measured as cell protein) was varied. The lowered net release of homocysteine with increasing cell density is probably due to a more extensive metabolism of intracellular homocysteine into methionine (remethylation) or into cysteine (transsulfuration) as described in the introduction. The correlation between lowered extracellular cysteine and increased cell protein can probably be attributed to increased consumption. The decrease of intracellular glutathione and increase of intracellular cysteine with increasing cell protein is, however, not obvious. None of these findings concerning cysteine or glutathione seem to give any further information about the export mechanism of homocysteine.

One of the conclusions of the experiment with cells incubated in the same medium for 1–3 days is that the cells are able to take up extracellularly released homocysteine, thereby decreasing the extracellular pool of homocysteine. This is in agreement with findings of a previous study [22], in which homocysteine added to medium, deficient in methionine or cysteine, is used in the intracellular metabolism where it to some extent replaces both methionine and cysteine. Another conclusion from these experiments is that the presence of copper ions maintained the large pool of extracellular homocysteine even on the third day. This might either be due to stimulation of *S*-adenosyl-homocysteine hydrolase [19,20,23,24] or to a decrease in the reusage of extracellular homocysteine. It has been reported that cells in culture possess two transport systems for homocysteine, the well-known systems ASC and L [25]. The uptake of homocysteine was inhibited by cysteine and inhibition was also evident in the presence of a thiol-blocking reagent [25]. Thus, it is mainly homocysteine in its reduced form that is transported. The presence of copper ions increases the oxidative stress in cell cultures thereby decreasing the proportions of reduced thiol species extracellularly [19]. It is, therefore, possible that the reuptake of homocysteine in the presence of copper ions is lowered.

Cysteine and glutathione were also determined in the experiments with cells cultured in the same medium for 1–3 days in an attempt to further elucidate the metabolism of homocysteine. However, the pattern of these thiols did not seem to give any further information on the regulation of homocysteine release. It seems as if copper ions increased the total glutathione release (the sum of extracellular glutathione and cysteinylglycine). Cysteinylglycine is probably degraded to cysteine, which could be one explanation for the increased extracellular cysteine observed in the presence of copper ions. Another explanation could be a lowered uptake of cysteine due to the fact that copper ions decrease the concentration of cysteine, which is oxidized to cystine [19] and thus not taken up by cysteine transport systems [25]. The lowering of intracellular cysteine in the presence of copper ions might thus be due to a lowered uptake or an increased demand for cysteine because of increased synthesis of glutathione [20] and possibly also metallothioneine [26]. Thus, it seems as if the copper effect upon the metabolism of cysteine and glutathione does not explain the effect of copper ions on homocysteine release.

4.2. Findings in the different cell lines

The ratio between intracellular homocysteine and

the total amount of homocysteine is a measure of the ability of the cell to export the intracellularly produced homocysteine. The ratio also reflects the reuse of extracellular homocysteine. Since the endothelial cells show a very low concentration of intracellular homocysteine and a low ratio (intracellular/total homocysteine) compared to the other two human cell lines, it seems as if the endothelial cells under basal conditions export most of the intracellularly produced homocysteine. Furthermore, they do not metabolize extracellular homocysteine to the same extent as HeLa cells. This might be attributed to either a low reuptake capacity of exported homocysteine in endothelial cells or to that these cells only have a limited ability to metabolize homocysteine in the remethylation or transsulfuration pathways. However, after 3 days in the same medium, extracellular concentration of homocysteine in endothelial cell cultures was decreased compared to the concentration after 1 day of incubation. This indicates that some metabolism of extracellular homocysteine can occur in endothelial cells. The presence of copper ions in the 3 day experiment seemed to inhibit the reusage of homocysteine, probably by decreasing the concentration of reduced homocysteine and thereby lowering the uptake of homocysteine, as earlier discussed.

After addition of homocysteine, all cell lines exhibited similar ratios between intracellular homocysteine and total amount of homocysteine in the cell culture. Thus, the intracellular homocysteine concentration in endothelial cells is more influenced by the extracellular concentration of homocysteine than is the intracellular concentration in HeLa and hepatoma cells. In this context it is therefore of interest that Wang et al. [27] recently reported that low concentrations of homocysteine (10–50 μmol/l), but not cysteine, inhibited proliferation of endothelial cells without affecting other cell types. The inhibition of endothelial cell growth was associated with an increase of *S*-adenosyl-homocysteine level and reduced methylation [27]. The finding in the present study of a very low concentration of intracellular homocysteine in endothelial cells under basal conditions might be linked to their reported sensitivity [27] to increased extracellular homocysteine concentrations.

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